

Sense transformation reveals a novel role for class I β -1,3-glucanase in tobacco seed germination

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Summary

'Coat-enhanced' seed dormancy of many dicotyledonous species, including tobacco, is released during after-ripening. Rupture of the endosperm, which is the limiting step in tobacco seed germination, is preceded by induction of class I β -1,3-glucanase (β GLUI) in the micropylar endosperm where the radicle will penetrate. Treating after-ripened tobacco seeds with abscisic acid (ABA) delays endosperm rupture and inhibits β GLUI induction. Sense transformation with a chimeric ABA-inducible β GLUI transgene resulted in over-expression of β GLUI in seeds and promoted endosperm rupture of mature seeds and of ABA-treated after-ripened seeds. Taken together, these results provide direct evidence that β GLUI contributes to endosperm rupture. Over-expression of β GLUI during germination also replaced the effects of after-ripening on endosperm rupture. This suggests that regulation of β GLUI by ABA signalling pathways might have a key role in after-ripening.

Keywords: abscisic acid, after-ripening, endosperm-limited seed germination, gene function, β -1,3-glucanase, sense transformation.

Introduction

Little is known about the molecular basis for the after-ripening and germination of dicotyledonous seeds (Bewley, 1997a). During seed maturation, water content decreases, abscisic acid (ABA) accumulates, and primary dormancy is established (Hilhorst, 1995; Li and Foley, 1997; Rock and Quatrano, 1995). Primary dormancy of many species can be altered by after-ripening, i.e. the storage of mature seeds for several months under dry, warm conditions (Hilhorst, 1995; Kasperbauer, 1968; Koornneef and Karssen, 1994; Li and Foley, 1997). Under favourable conditions, imbibition of water by non-dormant seeds results in a burst of respiration, rapid growth of the embryo, rupture of the covering layers, and finally emergence of the radicle. Many of these seeds exhibit 'coat-enhanced' dormancy in which the emergence of the radicle is physically restrained by the covering layers (Baskin and Baskin, 1998; Bewley, 1997a; Hilhorst, 1995). In the case of endosperm-limited germination, it is believed that hydrolytic enzymes facilitate weakening of the endosperm surrounding the radicle tip by hydrolysing cell-wall materials (Bewley, 1997b; Black, 1996; Ni and Bradford, 1993; Sitrit *et al.*, 1999); however, direct evidence for this hypothesis from studies with transgenic seeds is lacking.

The first morphological event following imbibition of tobacco seeds is rupture of the seed coat (testa). This is followed by rupture of the endosperm, which is the limiting step of tobacco seed germination (Arcila and Mohapatra, 1983; Leubner-Metzger *et al.*, 1995). β -1,3-glucanase (β GLU) activity is induced after testa rupture and just prior to the onset of endosperm rupture. This activity is localized in the micropylar endosperm at the site where the radicle will emerge, and results from transcriptional induction of class I β -1,3-glucanase (β GLUI) (Leubner-Metzger *et al.*, 1995). The induction of β GLUI and endosperm rupture are tightly linked in response to physiological factors known to affect the incidence and timing of germination (Leubner-Metzger *et al.*, 1996; Leubner-Metzger *et al.*, 1998). For example, ABA treatment specifically delays endosperm rupture and inhibits the induction of β GLUI. Kinetic analysis of this effect strongly suggests that the onset of endosperm rupture depends on a critical threshold concentration of β GLUI.

In the present study, we established by sense transformation that β GLUI has a causal role in endosperm rupture. We also provide less direct evidence that after-ripening

could affect endosperm rupture, at least in part, by a β GLU-mediated process.

Results

High-level expression of β GLU1 can promote endosperm rupture

Tobacco plants were transformed with the expression vector pKSG7 carrying a chimeric tobacco β GLU1 gene regulated by the castor bean *Cat1* gene promoter. The *Cat1* promoter was chosen because it directs high-level expression of a reporter gene in the endosperm of germinating seeds of transgenic tobacco (Suzuki *et al.*, 1995). Unless indicated otherwise, after-ripened seeds obtained by self-fertilization of primary sense (TKSG7) and empty-vector (TCIB1) transformants were used. Seeds were imbibed in the presence of ABA to delay the onset of germination and inhibit the induction of host β GLU1 genes (Leubner-Metzger *et al.*, 1995).

In our initial screen, we scored the incidence of germination, i.e. percentage of endosperm rupture, at

73 h after the start of imbibition in the presence of $1\ \mu\text{M}$ ABA in continuous light. Under these conditions, the germination rate of the 27 empty-vector TCIB1 lines used as controls was $6.8 \pm 1.2\%$ (mean \pm SE) and the population of seeds contained a mean β GLU activity of 0.9 ± 0.1 pkat/seed. In contrast, 41.9% of the 43 TKSG7 lines germinated at rates at least twofold higher than the TCIB1 controls and exhibited a 7.5-fold higher mean β GLU activity. Thus, germination was correlated with elevated β GLU activity in numerous, independent sense transformants.

Four representative TKSG7 lines with high germination rates in the presence of $1\ \mu\text{M}$ ABA were chosen that differed in transgene dose and β GLU content. The time course for testa rupture, endosperm rupture and β GLU activity accumulation was determined for lines carrying one (TKSG7-43), two (TKSG7-34 and TKSG7-42) and three or more (TKSG7-10) transgene loci and for four control TCIB1 lines imbibed with and without $10\ \mu\text{M}$ ABA in the incubation medium. The time course for testa rupture was not affected by ABA treatment and was very similar for TKSG7 and TCIB1 seeds (data not shown). Figure 1(a) shows that after-ripened seed of TKSG7 and TCIB1 lines

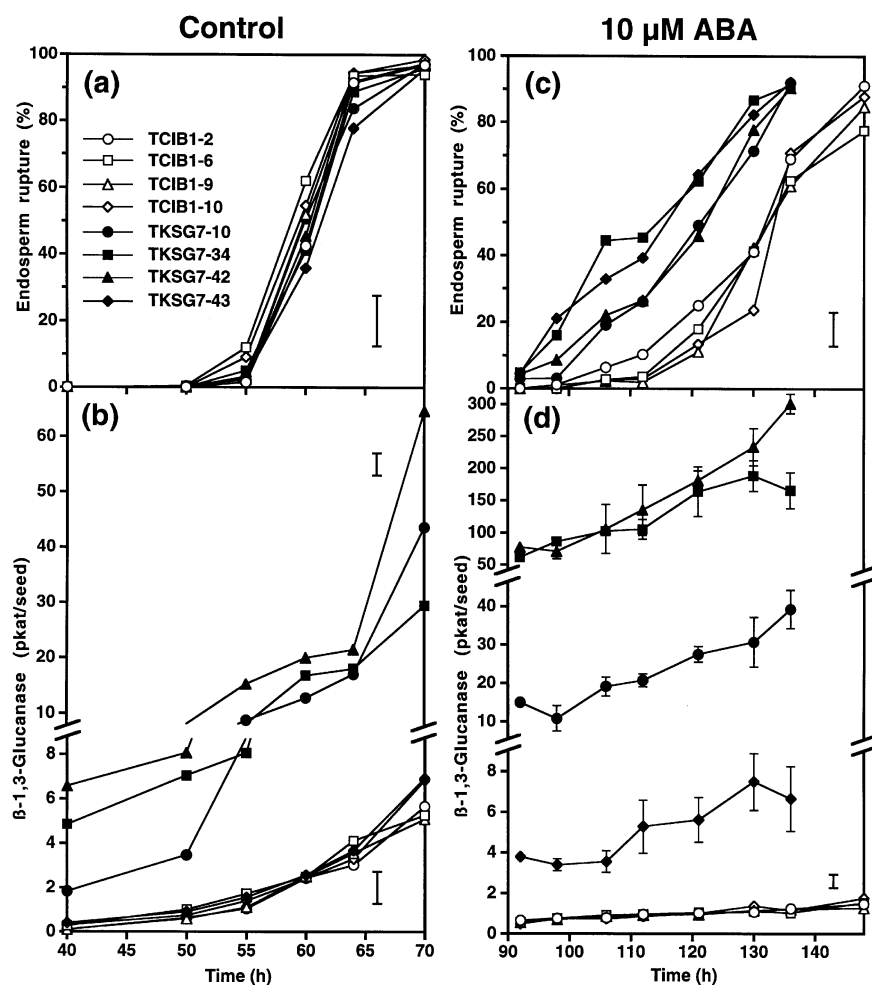


Figure 1. The effect of $10\ \mu\text{M}$ ABA on the time course of endosperm rupture and β GLU accumulation in after-ripened S_1 seeds of independent sense β GLU1 (TKSG7) and empty-vector (TCIB1) transformants. (a) and (c) The incidence of endosperm rupture expressed as the percentage of 100–150 seeds scored over time since the start of imbibition in continuous light without (a) and with (c) $10\ \mu\text{M}$ ABA added. Segregating seed populations of lines carrying one (TKSG7-43), two (TKSG7-34 and TKSG7-42) and three or more (TKSG7-10) transgene loci were used. The means of two replicates from one experiment are presented. Similar results were obtained in a second experiment. Error bars indicate the largest standard error value. (b) and (d) The β GLU enzyme activities of imbibed TKSG7 and TCIB1 seed populations without (b) and with (d) $10\ \mu\text{M}$ ABA. Error bars (b) indicate the largest standard error values of lines TKSG7-10, TKSG7-34 and TKSG7-42 (upper corner) and of lines TCIB1 and TKSG7-43 (lower corner). Error bars (d) indicate individual standard error values (TKSG7 lines) and the largest standard error value (TCIB1 lines). Note the difference in scale of (b) and (d).

did not differ in timing of endosperm rupture when imbibed in medium without added ABA. As reported earlier for wild-type seeds (Leubner-Metzger *et al.*, 1995), treatment with 10 μ M ABA dramatically delayed by approximately 70 h the time for 50% endosperm rupture of TCIB1 seeds (Figure 1c). This delay in endosperm rupture was substantially reduced by about 15 h in TKSG7 seeds.

Three of the TKSG7 lines accumulated considerably more β GLU activity than did the empty-vector control lines when imbibed without added ABA. The activity of line TKSG7-43 was comparable to the TCIB1 lines (Figure 1b). The finding that over-expression of β GLU in sense lines did not promote endosperm rupture under these conditions is consistent with studies showing that β GLU activities above approximately 5 pkat/seed at the onset of rupture do not increase the incidence of endosperm rupture (Leubner-Metzger *et al.*, 1995). ABA treatment inhibited β GLU accumulation of TCIB1 seeds (Figure 1d) as reported earlier for wild-type seed (Leubner-Metzger *et al.*, 1995). In contrast, ABA treatment markedly increased the β GLU activities of the TKSG7 seeds. Although the level of β GLU activity and the induction in response to 10 μ M ABA differed considerably for the different TKSG7 lines, even the least responsive line, TKSG7-43, showed at least fivefold higher β GLU activities relative to the TCIB1 controls throughout the experiment. Quantitative immunoblot analysis using antibodies that detect all known β GLU classes and RNA-blot hybridization using a probe specific for β GLU1 mRNA confirmed that the increased β GLU activity of the TKSG7 lines is due exclusively to increased amounts of β GLU1 (data not shown). Taken together, these findings indicate that expression of β GLU1 under the regulation of the *Cat1* promoter increases β GLU1

levels during endosperm rupture and results in a partial reversal of the delay in rupture due to ABA.

After-ripening and photodormancy effects

We compared germination in the light of 'fresh' seeds, i.e. mature seeds 40 days after pollination (DAP), and of after-ripened seeds stored at room temperature for at least 6 months after harvest. When imbibed without added ABA, essentially all tobacco seeds eventually germinate. Thus, changes in the onset of germination can be detected by measuring the germination rate at a fixed time after the start of imbibition. Table 1 shows that after-ripening increased the germination rate of wild-type and TCIB1-2 seed. This indicates that after-ripening can release dormancy of Havana 425 tobacco seeds. Fresh sense seeds (TKSG7) germinated at approximately 1.5-fold higher rates than did the controls, whereas after-ripened sense and control seeds germinated at comparable rates. The β GLU activity at the time of endosperm rupture was higher in after-ripened than in fresh control seeds. Independent of after-ripening, the β GLU content of sense-transformed seeds was higher than that of the control seeds. Taken together, these results suggest that over-expression of β GLU can replace the effect of after-ripening on endosperm rupture in the light.

Havana 425 tobacco seeds exhibit photodormancy, i.e. fresh seeds do not germinate in the dark, even after prolonged periods of time (Leubner-Metzger *et al.*, 1996). Table 1 shows that after-ripening contributes to the release of photodormancy and that this effect varies greatly for different seed batches as reported for other tobacco cultivars (Kasperbauer, 1968). Sense transformation did not have detectable effects on either photodormancy of

Table 1. Effect of after-ripening on endosperm rupture, β GLU activity and photodormancy of sense β GLU1 seeds

Lines ^a	Continuous light				Darkness	
	Endosperm rupture (%) ^b		β GLU (pkat/seed) ^c		Non-photodormancy (%) ^d	
	'Fresh' seed	After-ripened seed	'Fresh' seed	After-ripened seed	'Fresh' seed	After-ripened seed
Wild-type	49.8 \pm 5.2	79.2 \pm 6.8	2.4 \pm 0.1	3.2 \pm 0.2	0.4 \pm 0.2	50.3 \pm 16.5 (0.0–80.6)
TCIB1-2	49.7 \pm 2.1	81.4 \pm 5.2	2.2 \pm 0.1	2.9 \pm 0.2	0.3 \pm 0.2	40.0 \pm 12.6 (9.1–80.1)
TKSG7-32	74.4 \pm 2.1	79.0 \pm 2.4	9.1 \pm 0.2	5.3 \pm 0.2	0.3 \pm 0.3	29.7 \pm 13.5 (3.0–63.6)
TKSG7-38	77.1 \pm 2.1	73.5 \pm 0.1	8.9 \pm 0.2	4.9 \pm 0.3	0.9 \pm 0.2	39.0 \pm 18.9 (0.0–82.4)
TKSG7-43	75.3 \pm 3.6	75.8 \pm 7.4	8.2 \pm 0.5	4.7 \pm 0.4	0.4 \pm 0.2	43.6 \pm 19.6 (9.4–90.2)

^aWild type and independent, homozygous, monogenic vector-control (TCIB1) and sense β GLU1 (TKSG7) tobacco lines selected from the S₂ seed generation. ^bMean \pm SE of 'fresh' (directly after harvest) and after-ripened (\geq 6 months of dry storage) seed from 3–6 capsules scored 72 h and 67 h, respectively, after the start of imbibition in continuous light in control medium without antibiotics. ^cMean \pm SE β GLU activities of protein extracts prepared from seed samples described under ^b. ^dMean \pm SE of 'fresh' and after-ripened seed from 3–6 capsules scored after 10 days incubation in the dark in control medium without antibiotics. The range obtained with different capsules is shown in parentheses.

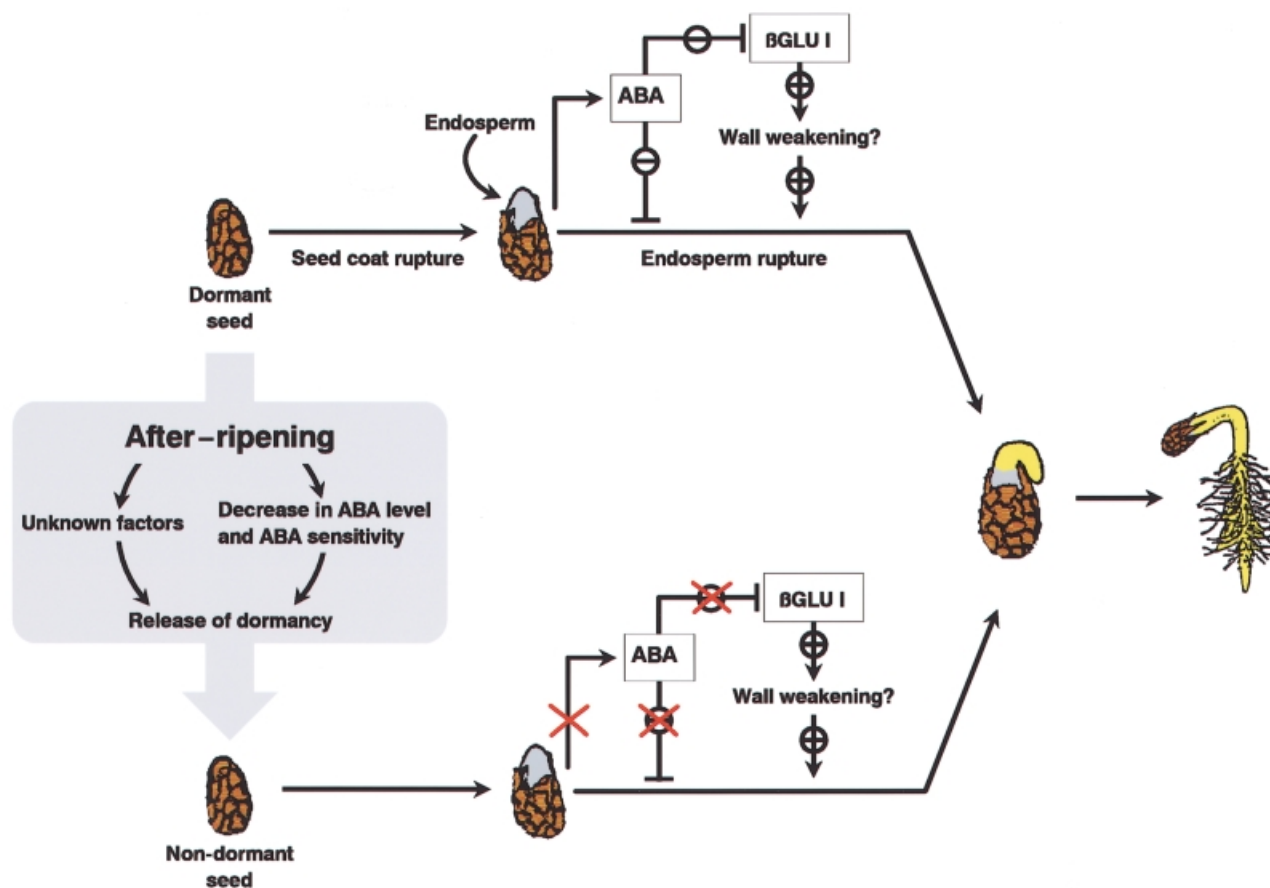


Figure 2. A speculative model relating after-ripening, β GLU I and endosperm rupture.

According to the model, β GLU I, which is transcriptionally down-regulated by ABA, contributes to endosperm wall weakening and breaking of coat-enhanced dormancy. ABA accumulated during seed maturation is sufficient to delay β GLU I induction and endosperm rupture. After-ripening decreases ABA levels and possibly sensitivity to ABA, allowing the induction of β GLU I during imbibition, which helps modulate coat-enhanced dormancy. The model does not exclude the possibility that other factors contribute to after-ripening and modulation of coat-enhanced dormancy, or that ABA has additional functions, e.g. in photodormancy.

fresh seed or on the release of photodormancy due to after-ripening.

Discussion

β GLU I contributes to endosperm rupture

β GLU I induction in the micropylar endosperm is tightly correlated with endosperm rupture under a variety of physiological conditions that delay or promote tobacco-seed germination (reviewed in Leubner-Metzger and Meins, 1999). In the present study, we provide direct evidence that β GLU I has a role in endosperm rupture. The castor bean *Cat1* promoter shown earlier to confer micropylar-endosperm expression in tobacco seeds (Suzuki *et al.*, 1995) was used to regulate expression of a β GLU I sense transgene. Expression of maize and *N. plumbaginifolia* orthologues of the castor bean *Cat1* gene is strongly induced by ABA during seed maturation and germination (Bueno *et al.*, 1998; Guan and Scandalios,

1993; Willekens *et al.*, 1994; Williamson and Scandalios, 1992). We found that ABA treatment induces the castor bean *Cat1* promoter in tobacco seeds. This effect was exploited to achieve high-level β GLU I expression in seeds treated with ABA to delay endosperm rupture and block host β GLU I expression. The results indicate that expression of β GLU I reduced the delay in endosperm rupture of after-ripened TKS7 seeds due to ABA treatment, but did not affect the onset of testa rupture. Increased β GLU I expression also promoted germination of fresh TKS7 seeds that were not treated with ABA. Taken together, these results show that β GLU I substantially contributes to endosperm rupture, which is the limiting step in tobacco seed germination.

We also attempted to block β GLU I induction during endosperm rupture by antisense transformation using the *Cat1* promoter. As is often the case for antisense experiments, this approach was not successful. Although numerous independent transformants were screened, none were found with reduced β GLU activity, β GLU I

protein or β GLU1 mRNA levels, and none showed effects on endosperm rupture.

In cases of endosperm-limited germination, radicle emergence depends on both wall weakening and sufficient growth of the embryo to overcome the mechanical resistance of the endosperm (Bewley, 1997b; Ni and Bradford, 1993). Ultrastructural studies suggest that the endospermic hole formed at the micropylar end of tobacco seeds results from endosperm 'dissolution' rather than from the 'pushing' action of radicle growth (Arcila and Mohapatra, 1983). Earlier studies showed that ABA treatment of wild-type tobacco seeds delayed the onset of endosperm rupture and inhibited β GLU1 induction in a dose-dependent fashion (Leubner-Metzger *et al.*, 1995). Independent of the ABA concentration, the onset of endosperm rupture was correlated with a β GLU activity of roughly 2 pkat/seed, and the incidence of endosperm rupture did not increase with β GLU activities greater than roughly 5 pkat/seed. In the present experiment, the onset of endosperm rupture of control TCIB1 seeds treated with 10 μ M ABA was somewhat lower, roughly 1 pkat/seed. The important point is that all independent sense lines exhibited earlier germination and β GLU activities above this threshold value. This further supports the hypothesis that a threshold β GLU content is required for endosperm rupture, and suggests that accumulation of β GLU is necessary but not sufficient for endosperm weakening.

The mechanism underlying the effect of β GLU1 on endosperm weakening is not known. One possibility is that β GLU1 helps to hydrolyse extracellular polysaccharides important for strengthening the endosperm wall. This would imply that β GLU1, which is a vacuolar protein (Keefe *et al.*, 1990), can be alternatively targeted for secretion. Recent studies suggesting that tobacco β GLU1 (Kunze *et al.*, 1998) and other vacuolar proteins (Kjemtrup *et al.*, 1995) can be secreted support this view. A possible target for β GLU1 is callose, which is a substrate for the enzyme (Stone and Clarke, 1992). Callose has been found in the impermeable covering layers of seeds of some dicotyledonous species, e.g. *Cucumis melo* (Welbaum *et al.*, 1995; Yim and Bradford, 1998), but not in others, e.g. tomato and pepper (Beresniewicz *et al.*, 1995). The presence of callose or other potential substrates of β GLU in the endosperm of tobacco has not been demonstrated and is an interesting area for further investigation.

The effect of after-ripening on endosperm could involve β GLU1

We found that after-ripening of Havana 425 tobacco seed had two effects on germination: it breaks photodormancy, which blocks germination prior to testa rupture (Leubner-

Metzger *et al.*, 1996; Mohapatra and Johnson, 1978), and it modulates coat-enhanced dormancy, which involves endosperm rupture. Little is known about the molecular basis for dormancy or the modulation of dormancy during after-ripening (Bewley, 1997a; Li and Foley, 1997). Although several genes that are differentially expressed in imbibed dormant and non-dormant seeds have been identified, none have been shown to be directly involved in the maintenance or breaking of dormancy (Bewley, 1997a; Li and Foley, 1997). Over-expression of β GLU1 during germination replaced the after-ripening effect on endosperm rupture, but did not influence photodormancy. Endogenous production of ABA is needed to establish and maintain dormancy in many species including tobacco (Artsaenko *et al.*, 1995; Bewley, 1997a; Fry *et al.*, 1999; Grappin *et al.* 2000; Koornneef and Karssen, 1994; Li and Foley, 1997; Rock and Quatrano, 1995). The onset of dormancy in tobacco is correlated with a peak in ABA content, which declines rapidly during further seed maturation (approximately 15–25 DAP) (Jiang *et al.*, 1996; Phillips *et al.*, 1997; Yamaguchi-Shinozaki *et al.*, 1990). The fresh tobacco seeds we used were sampled at approximately 40 DAP, which is after maturation and establishment of primary dormancy is complete. After-ripening is often correlated with a further decline in ABA content and decreased sensitivity to ABA (Benech-Arnold *et al.*, 1999; Bewley, 1997a; Grappin *et al.* 2000; Hilhorst, 1995; Li and Foley, 1997; Ren and Kermodé, 1999).

Recent work with *Nicotiana plumbaginifolia* suggests that *de novo* synthesis of ABA in imbibed fresh seed also contributes to dormancy (Grappin *et al.* 2000). ABA treatment at concentrations in the range found in mature wild-type tobacco seeds transcriptionally down-regulates β GLU1 expression in the micropylar endosperm and markedly delays endosperm rupture (Leubner-Metzger *et al.*, 1995). Moreover, expression of host β GLU1 in control seeds, which is down-regulated by ABA, was increased by after-ripening; whereas expression of the *Cat1*-regulated β GLU1 gene in sense seeds, which is up-regulated by ABA, was decreased by after-ripening (Table 1). This leads us to speculate that the expression of β GLU1 needed for endosperm weakening is inhibited by ABA present in the fresh control seed. During after-ripening, decreasing ABA levels and decreasing sensitivity to ABA eventually permit β GLU1 expression, which results in the release of dormancy (Figure 2).

Experimental procedures

Plasmid construction and plant transformation

The chimeric sense- β GLU1 construct KSG7 was obtained by transcriptional fusion between the 2.7 kb *EcoRI*–*Bam*HI genomic DNA fragment of pCBC1 that contains the promoter of the castor

bean *Cat1* gene (Suzuki *et al.*, 1994; Suzuki *et al.*, 1995) and the 2.9 kb *Bam*HI–*Sph*I genomic DNA fragment of the β GLU1B (*Glb*) gene which contains an introduced *Bam*HI site located at the transcription start site, the entire coding sequence, the intron and 0.9 kb of 3' flanking region (Hart *et al.*, 1993). The resulting 5.6 kb *Eco*RI–*Sph*I DNA fragment replaced the cauliflower mosaic virus (CaMV) 35S promoter in the pDH51 vector (Pietrzak *et al.*, 1986) and is terminated by the CaMV 35S RNA polyadenylation signal. The binary vector pCIB200 (Neuhaus *et al.*, 1992), which carries the chimeric neomycin phosphotransferase gene (*NptII*) under the control of the nopaline synthase (*Nos*) promoter and terminator, was linearized in the polylinker with *Kpn*I and *Eco*RI and ligated to the *Kpn*I–*Eco*RI fragment that contained the chimeric sense- β GLU1 gene. The resulting expression vector pKSG7 carried the chimeric sense- β GLU1 and *Nos*–*NptII* genes transcribed in the same direction.

The methods for introducing the pCIB200 expression vectors into *Agrobacterium tumefaciens*, Ti-plasmid transformation of *Nicotiana tabacum* L. cv. Havana 425 leaf discs, regeneration of plants, and segregation tests using the kanamycin resistance marker have been described previously (Beffa *et al.*, 1993). TCIB1 transformants obtained with the empty-vector plasmid pCIB200 were used as controls. Segregation tests were performed with S₁ seeds obtained by self-fertilization of independent primary transformants. Homozygous, monogenic S₂ seeds were obtained by self-fertilization of S₁ plants using kanamycin resistance as the marker.

Germination experiments

Seed from mature capsules of wild-type or transformed Havana 425 tobacco were used either at approximately 40 DAP (fresh seed) or after at least 6 months of dry storage at room temperature (after-ripened seed), as indicated. Germination analyses were performed as described by Leubner-Metzger *et al.* (1998). In brief, 100–150 seeds were sown on plastic Petri dishes containing filter paper wetted with a nutrient solution supplemented as indicated with 50 μ g ml⁻¹ kanamycin, 100 μ g ml⁻¹ Claforan (Hoechst-Pharma AG, Zürich, Switzerland), and 1 or 10 μ M *cis*-(\pm)-abscisic acid (ABA). Petri dishes were incubated at 25°C in continuous white light (3000 lux, Philips 'TL'D 35W/33 lamps) or in darkness. After scoring for germination, seeds were stored at –80°C for subsequent analysis.

Analysis of proteins and RNA

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis, protein determination, preparation of total RNA and RNA-blot hybridization have been described previously (Leubner-Metzger *et al.*, 1995). In brief, β GLU activity was assayed radiometrically using [³H]-laminarin as the substrate. The rabbit anti-tobacco β GLU1 antibody used for immunoblot analysis detects the class I, class II and class III isoforms of the enzyme (Beffa *et al.*, 1993; Neuhaus *et al.*, 1992). The 'ECF Western blotting system' (Amersham Pharmacia Biotech, Amersham, UK) was used for quantitative immunoblot analysis. The DNA probes used for RNA-blot hybridization were the 1 kb *Pst*I fragment of the tobacco β GLU1 cDNA pGL43 (Shinshi *et al.*, 1988) and the 1.8 kb *Eco*RI fragment of genomic DNA encoding tomato 18S ribosomal RNA (Schmidt-Puchta *et al.*, 1989). Hybridized membranes were washed at high stringency. Signals were detected and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA,

USA) and corrected for RNA loading based on the 18S ribosomal RNA signal.

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References

- Arcila, J. and Mohapatra, S.C. (1983) Development of tobacco seedling. 2. Morphogenesis during radicle protrusion. *Tobacco Sci.* **27**, 35–40.
- Artsaenko, O., Peisker, M., zur Nieden, U., Fiedler, U., Weiler, E.W., Müntz, K. and Conrad, U. (1995) Expression of a single-chain Fv antibody against abscisic acid creates a wilted phenotype in transgenic tobacco. *Plant J.* **8**, 745–750.
- Baskin, C.C. and Baskin, J.M. (1998) *Seeds – Ecology, Biogeography, and Evolution of Dormancy and Germination*. San Diego: Academic Press.
- Beffa, R.S., Neuhaus, J.-M. and Meins, F. Jr (1993) Physiological compensation in antisense transformants: specific induction of an 'ersatz' glucan endo-1,3- β -glucosidase in plants infected with necrotizing viruses. *Proc. Natl Acad. Sci. USA*, **90**, 8792–8796.
- Benech-Arnold, R.L., Giallorenzi, M.C., Frank, J. and Rodriguez, V. (1999) Termination of hull-imposed dormancy in developing barley grains is correlated with changes in embryonic ABA levels and sensitivity. *Seed Sci. Res.* **9**, 39–47.
- Beresniewicz, M.M., Taylor, A.G., Goffinet, M.C. and Koeller, W.D. (1995) Chemical nature of a semipermeable layer in seed coats of leek, onion (Liliaceae), tomato and pepper (Solanaceae). *Seed Sci. Tech.*, **23**, 135–145.
- Bewley, J.D. (1997a) Seed germination and dormancy. *Plant Cell*, **9**, 1055–1066.
- Bewley, J.D. (1997b) Breaking down the walls – a role for endo- β -mannanase in release from seed dormancy? *Trends Plant Sci.* **2**, 464–469.
- Black, M. (1996) Liberating the radicle: a case for softening-up. *Seed Sci. Res.* **6**, 39–42.
- Bueno, P., Piqueras, A., Kupera, J., Saviouré, A., Verbruggen, N., van Montagu, M., Inzé, D. (1998) Expression of antioxidant enzymes in response to abscisic acid and high osmoticum in tobacco BY-2 cell cultures. *Plant Sci.* **138**, 27–34.
- Fry, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. (1999) Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. *Plant Mol. Biol.* **39**, 1267–1274.
- Grappin, P., Bouinot, D., Sotta, B., Miginiac, E. and Jullien, M. (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta*, **210**, 279–285.
- Guan, L. and Scandalios, J.G. (1993) Characterization of the catalase antioxidant defense gene *Cat1* of maize, and its developmentally regulated expression in transgenic tobacco. *Plant J.* **3**, 527–536.
- Hart, C.M., Nagy, F. and Meins, F., Jr (1993) A 61 bp enhancer element of the tobacco β -1,3-glucanase B gene interacts with one or more regulated nuclear proteins. *Plant Mol. Biol.* **21**, 121–131.

- Hilhorst, H.W.M.** (1995) A critical update on seed dormancy. I. Primary dormancy. *Seed Sci. Res.* **5**, 61–73.
- Jiang, L., Abrams, S.R. and Kermod, A.R.** (1996) Vicilin and napin storage-protein gene promoters are responsive to abscisic acid in developing tobacco seed but lose sensitivity following premature desiccation. *Plant Physiol.* **110**, 1135–1144.
- Kasperbauer, M.J.** (1968) Dark-germination of reciprocal hybrid seed from light-requiring and -indifferent *Nicotiana tabacum*. *Physiol. Plant.* **21**, 1308–1311.
- Keefe, D., Hinz, U. and Meins, F., Jr** (1990) The effect of ethylene on the cell-type-specific and intracellular localization of β -1,3-glucanase and chitinase in tobacco leaves. *Planta*, **182**, 43–51.
- Kjemtrup, S., Borkhsenius, O., Raikhel, N.V. and Chrispeels, M.J.** (1995) Targeting and release of phytohemagglutinin from the roots of bean seedlings. *Plant Physiol.* **109**, 603–610.
- Koorneef, M. and Karssen, C.M.** (1994) Seed dormancy and germination. In *Arabidopsis* (Meyerowitz, E.M. and Somerville, C.R., eds). Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 313–334.
- Kunze, I., Kunze, G., Bröker, M., Manteuffel, R., Meins, F. Jr and Müntz, K.** (1998) Evidence for secretion of vacuolar α -mannosidase, class I chitinase, and class I β -1,3-glucanase in suspension cultures of tobacco cells. *Planta*, **205**, 92–99.
- Leubner-Metzger, G. and Meins, F. Jr** (1999) Functions and regulation of plant β -1,3-glucanases (PR-2). In *Pathogenesis-Related Proteins in Plants* (Datta, S.K. and Muthukrishnan, S., eds). Boca Raton: CRC Press, pp. 49–76.
- Leubner-Metzger, G., Fründt, C., Vögeli-Lange, R. and Meins, F. Jr** (1995) Class I β -1,3-glucanase in the endosperm of tobacco during germination. *Plant Physiol.* **109**, 751–759.
- Leubner-Metzger, G., Fründt, C. and Meins, F. Jr** (1996) Effects of gibberellins, darkness and osmotica on endosperm rupture and class I β -1,3-glucanase induction in tobacco seed germination. *Planta*, **199**, 282–288.
- Leubner-Metzger, G., Petruzelli, L., Waldvogel, R., Vögeli-Lange, R. and Meins, F. Jr** (1998) Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I β -1,3-glucanase during tobacco seed germination. *Plant Mol. Biol.* **38**, 785–795.
- Li, B.L. and Foley, M.E.** (1997) Genetic and molecular control of seed dormancy. *Trends Plant Sci.* **2**, 384–389.
- Mohapatra, S.C. and Johnson, W.H.** (1978) Development of the tobacco seedling. 1. Relationship between moisture uptake and light sensitivity during seed germination in a flue-cured variety. *Tobacco Res.* **4**, 41–49.
- Neuhaus, J.M., Flores, S., Keefe, D., Ahl, G.P. and Meins, F. Jr** (1992) The function of vacuolar β -1,3-glucanase investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvestris* plants to *Cercospora nicotianae* infection. *Plant Mol. Biol.* **19**, 803–813.
- Ni, B.R. and Bradford, K.J.** (1993) Germination and dormancy of abscisic acid-deficient and gibberellin-deficient mutant tomato (*Lycopersicon esculentum*) seeds – sensitivity of germination to abscisic acid, gibberellin, and water potential. *Plant Physiol.* **101**, 607–617.
- Phillips, J., Artsaenko, O., Fiedler, U., Horstmann, C., Mock, H.P., Müntz, K. and Conrad, U.** (1997) Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. *EMBO J.* **16**, 4489–4496.
- Pietrzak, M., Shillito, R.D., Hohn, T. and Potrykus, I.** (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. *Nucl. Acids Res.* **14**, 5857–5868.
- Ren, C. and Kermod, A.R.** (1999) Analyses to determine the role of the megagametophyte and other seed tissues in dormancy maintenance of yellow cedar (*Chamaecyparis nootkatensis*) seeds: morphological, cellular and physiological changes following moist chilling and during germination. *J. Exp. Bot.* **50**, 1403–1419.
- Rock, C.D. and Quatrano, R.S.** (1995) The role of hormones during seed development. In *Plant Hormones* (Davies, P.J., ed). Dordrecht: Kluwer Academic Publishers, pp. 671–697.
- Schmidt-Puchta, W., Kütemeier, G., Günther, I., Haas, B. and Sängler, H.L.** (1989) Cloning and sequence analysis of the 18S ribosomal RNA gene of tomato and a secondary structure model of the 18S rRNA of angiosperms. *Mol. Gen. Genet.* **219**, 17–25.
- Shinshi, H., Wenzler, H., Neuhaus, J.M., Felix, G., Hofsteenge, J. and Meins, F. Jr** (1988) Evidence of amino and carboxyl-terminal processing of a plant defense-related enzyme primary structure of tobacco prepro- β -1,3-glucanase. *Proc. Natl Acad. Sci. USA*, **85**, 5541–5545.
- Sitrit, Y., Hadfield, K.A., Bennett, A.B., Bradford, K.J. and Downie, A.B.** (1999) Expression of a polygalacturonase associated with tomato seed germination. *Plant Physiol.* **121**, 419–428.
- Stone, B.A. and Clarke, A.E.** (1992) *Chemistry and Biology of (1,3)- β -Glucans*. Victoria, Australia: La Trobe University Press.
- Suzuki, M., Ario, T., Hattori, T., Nakamura, K. and Asahi, T.** (1994) Isolation and characterization of two tightly linked catalase genes from castor bean that are differentially regulated. *Plant Mol. Biol.* **25**, 507–516.
- Suzuki, M., Miyamoto, R., Hattori, T., Nakamura, K. and Asahi, T.** (1995) Differential regulation of the expression in transgenic tobacco of the gene for β -glucuronidase under the control of the 5'-upstream regions of two catalase genes from castor bean. *Plant Cell Physiol.* **36**, 273–279.
- Welbaum, G.E., Muthui, W.J., Wilson, J.H., Grayson, R.L. and Fell, R.D.** (1995) Weakening of muskmelon perisperm envelope tissue during germination. *J. Exptl. Bot.* **46**, 391–400.
- Willekens, H., Langebartels, C., Tiré, C., van Montagu, M., Inzé, D. and van Camp, W.** (1994) Differential expression of catalase genes in *Nicotiana plumbaginifolia* (L.). *Proc. Natl Acad. Sci. USA*, **91**, 10450–10454.
- Williamson, J.D. and Scandalios, J.G.** (1992) Differential response of maize catalases to abscisic acid: Vp1 transcriptional activator is not required for abscisic acid-regulated Cat1 expression. *Proc. Natl Acad. Sci. USA*, **89**, 8842–8846.
- Yamaguchi-Shinozaki, K., Mino, M., Mundy, J. and Chua, N.-H.** (1990) Analysis of an ABA-responsive rice gene promoter in transgenic tobacco. *Plant Mol. Biol.* **15**, 905–912.
- Yim, K.O. and Bradford, K.J.** (1998) Callose deposition is responsible for apoplastic semipermeability of the endosperm envelope of muskmelon seeds. *Plant Physiol.* **118**, 83–90.